DNA Structures

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i-Motif Formation with Locked Nucleic Acid (LNA)**

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The i-motif is formed by DNA stretches of two or more cytidine residues as present in the telomeric regions of human chromosomes and consists of criss-cross intercalated hemiprotonated C:C$^+$ base pairs.[1] The opposed dipoles of the exocyclic carbonyl and amino groups of the cytosine bases stabilize an antiparallel strand orientation between the parallel C:C$^+$ ladders.[1,2] The i-motif topology has four grooves, two of which are wide and two of which are narrow.[2,3] With a fairly low base-pair rise of approximately 3.1 Å and a small right-handed helical twist (8–28°), the i-motif has a compact structure. Sugar puckers are predominantly of the C3′-*endo* type in i-motif structures.[4] The i-motif structure is stabilized by hydrogen bonding, stacking, and dipole–dipole interactions, as well as by van der Waals interactions, and possibly also by C–H···O sugar "zippers" in the narrow grooves.[3,5] However, the actual energetic contribution of the C–H···O interactions is a matter of debate.[6,7] Circumstantial evidence for the interaction of proteins with i-motif structures indicate a possible biological role of i-motifs.[8–10]

Generally, few alterations in the DNA structure are tolerated in an i-motif. Most alterations destabilize the structure or prevent its formation altogether. Modifications with 3′-*S*-phosphorothiolate linkages can lead to an increase in thermostability of approximately 7–8 °C,[11] as they contain a preorganized C3′-*endo* sugar pucker. Like 3′-*S*-phosphorothiolates, RNA features C3′-*endo* sugar puckers. However, although RNA forms an i-motif structure, it is destabilized relative to the DNA i-motif. This destabilization is probably due to steric clashes in the narrow groove caused by the 2′-hydroxy group,[12,13] an assumption supported by the complete inability of 2′-*O*-methyl-modified oligonucleotides to form an i-motif, even when only one modification is introduced.[13]

A more drastic nucleic acid modification is peptide nucleic acid (PNA), in which the sugar-phosphate backbone is replaced by a polyamide backbone. Thus, PNA lacks the sugar moiety completely and has an uncharged backbone. Recent reports show that PNA is able to form both tetrameric PNA i-motifs[14,15] and hybrid DNA/PNA i-motifs.[16] Such structures could have applications within the field of nanobiotechnology and also serve as model systems for the assessment of the contribution of sugar–sugar contacts to i-motif formation.

Locked nucleic acid (LNA; Figure 1) is a conformationally constrained RNA mimic with a 2′-*O*–4′-*C* methylene bridge that locks the nucleotide analogue in a C3′-*endo* sugar

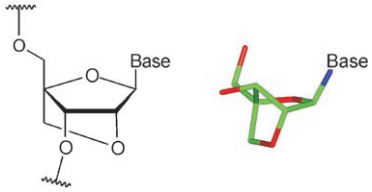

Figure 1. The chemical structure of LNA.

conformation. Generally, the introduction of LNA nucleotide monomers increases the thermal stability of DNA and RNA duplexes and triplexes.[17] LNA has the advantage over other nucleic acid analogues that it is biologically stable without being associated with toxic effects.[17] Recently, G-quadruplex formation with LNA was reported.[18,19] Herein, we report successful i-motif formation with LNA-modified TC_5 oligonucleotides at low pH values. i-Motif formation was monitored by CD, UV, and NMR spectroscopy (Table 1).

Table 1: Thermal denaturation of LNA i-motif structures.

Oligonucleotide (5′–3′)[a]		T_m [°C][b]	Populations[c]
ON0	d(TCCCCC)	43	100
ON1	d(T**C**CCCC)	43	58, 42
ON2	d(TCC**C**C)	49	100
ON3	d(T**C**C**C**C)	42	58, 28, 11, 3
ON4	d(TC**C**C**C**)	42	76, 16, 8
ON5	d(T**C**C**C**C)	–	

[a] **C** denotes an LNA-modified C nucleotide. [b] Melting temperatures (T_m values) were measured as the maximum of the first derivative of the CD melting curve (CD_{287} versus temperature; 10–70 °C; increase of 0.2 °C/min), which was recorded in Robinson–Britton buffer (10 mM, pH 4) with NaCl (100 mM). [c] The relative population of each i-motif conformation adopted by the oligonucleotides, as determined by ^{1}H NMR spectroscopy from the intensities of the thymine imino signals at $\delta \approx 11$ ppm.

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The formation and melting of LNA i-motifs was monitored at pH 4 by CD spectroscopy (Figure 2). Characteristic CD signatures (a positive peak at about 285 nm and a

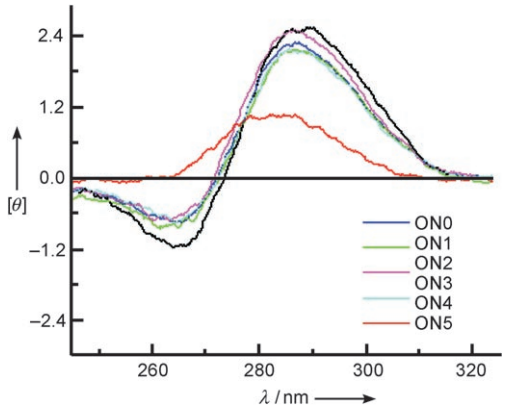

Figure 2. CD spectra of **ON0**–**ON5** (10 μM) in Robinson–Britton buffer (10 mM, pH 4) with NaCl (100 mM) at 15 °C. [θ] is molar ellipticity (10^6 deg cm^2 dmol^{-1}).

negative peak at about 265 nm) show that oligonucleotides **ON1**–**ON4**, which have been modified partly with LNA, form i-motif structures, whereas the almost fully modified LNA oligonucleotide **ON5** does not. Moreover, the resemblance of the CD spectra of **ON1**–**ON4** with the spectrum of **ON0**, the DNA i-motif, hints at only moderate structural differences between the LNA and DNA i-motifs. The CD melting curves of the oligonucleotides at pH 4 show that the melting temperatures of the LNA-modified oligonucleotides **ON1**, **ON3**, and **ON4** are similar to that of the unmodified oligonucleotide **ON0**, whereas **ON2** has a higher melting temperature ($\Delta T_m = 6$ °C) (Table 1). This result implies that LNA modifications are tolerated in an i-motif context, and that thermal stabilization is possible if the LNA-modified oligonucleotide is designed appropriately.

We carried out pH-dependent UV titrations to determine the pK_a value of the oligonucleotides that form i-motifs. The modified oligonucleotides **ON1**–**ON4** had a similar pK_a value of approximately 4.3, which is slightly lower than that of the DNA i-motif **ON0** (p$K_a = 4.6$). This decrease in the pK_a value probably stems from a lower intrinsic pK_a value of the LNA-C monomer relative to that of the DNA-C monomer, although structural factors could also play a part.

Chemical shifts of imino hydrogen atoms in the range $\delta =$ 15–16 ppm are excellent markers for protonated and hydrogen-bonded cytosine bases. One-dimensional ^{1}H NMR spectra recorded in H_2O confirm the ability of **ON1**–**ON4** to form i-motifs with C:C$^+$ base pairing. Only three signals that correspond to a C$^+$ imino proton were observed for the DNA i-motif **ON0**.[2] However, the spectra of **ON1**, **ON3**, and **ON4** display more than three signals in the range $\delta = 15$–16 ppm, which suggests that more than one intercalation topology is present. The thymine imino signals also indicate the presence of two or more topologies in slow exchange (Table 1). Interestingly, the spectrum of **ON2** displays only three signals due to protonated cytosine residues and one thymine signal and therefore indicates the presence of a single conformation for this tetraplex.

A comparison of NOESY spectra of **ON0** and **ON2** enabled the assignment of resonances and the identification of key NOE contacts peculiar to i-motif formation (see the Supporting Information). In particular, H1′–H1′ and imino–imino NOEs between nonsequential H1′ atoms and imino protons confirm the formation of the criss-cross intercalated tetrameric i-motif structure. The H1′–H1′ contacts are consistent with the base-stacking order T1-C6-C2-C5-C3-C4, which is also observed for the DNA i-motif **ON0**.[2]

i-Motif structures are composed of alternating "face-to-face" (ff) and "back-to-back" (bb) steps as defined with respect to the stacking of the face (5′ side) or back (3′ side) of the nucleobases (Figure 3). In ff steps, the 4′-O oxygen atoms are oriented towards one another in the narrow groove, whereas in bb steps, the hydrophobic edges (C3′–C4′) of the deoxyribose units are closer to one another. The OCH_2 moiety of LNA nucleotides spans the sugar from C2′ to C4′ and is placed in the narrow groove with the oxygen atom pointing towards the opposite strand in bb steps and with one of the CH_2 hydrogen atoms protruding into the groove in ff steps (Figure 3).

As i-motif formation is abolished completely with 2′-*O*-methyl-RNA, the ability of LNA to form an i-motif is

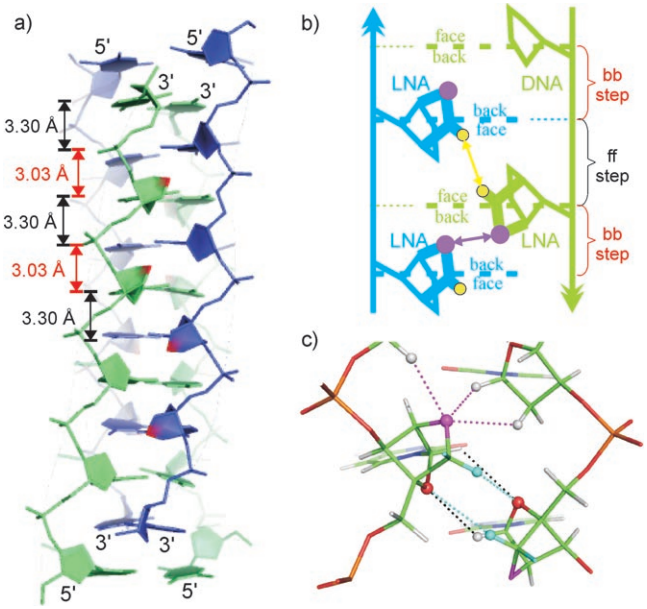

Figure 3. a) View into the narrow groove of the simulated **ON2** structure. LNA 2′-O atoms are shown in red. Average stacking distances are indicated for ff (black) and bb (red) steps. b) Schematic drawing of interactions between LNA–LNA sugar pairs. Bases are shown as dashed lines with back and face sides indicated. LNA 2′-O and 6′-H atoms are shown as magenta and yellow spheres, respectively. Arrows indicate steric clashes. c) Narrow-groove C–H···O network in the C3–**C4*** bb and **C4***–**C4** ff steps. LNA 2′-O, LNA 6′-H, 4′-O, and hydrogen atoms are represented as magenta, cyan, red, and white spheres, respectively. Contacts also observed in DNA i-motifs are shown with dashed black lines; contacts formed with LNA 2′-O (bb steps) and LNA 6′-H (ff steps) are shown with dashed magenta and cyan lines, respectively.

intriguing. We intend to explore further the structural basis for the formation of the LNA i-motif by NMR spectroscopy. However, to get an early impression of a probable LNA i-motif structure, we performed molecular dynamics (MD) simulations of the intercalation topology of **ON2**.

In general, the LNA i-motif is rather similar to the simulated structure of the DNA i-motif with regard to the stacking distance and stacking angle between C:C^+ pairs, as indicated experimentally by the similarity of the CD spectra. The most distinct difference between the two structures is the C–H···O network formed in the narrow groove. In DNA i-motifs, no C–H···O contacts are possible in bb steps. However, in the LNA i-motif, the LNA OCH_2 bridge extends the network to include bb steps (Figure 3). The extension of the C–H···O network does not alter the structure dramatically. For example, the width of the narrow groove is only slightly greater in the LNA i-motif (3.2 Å) than in the DNA i-motif (3.1 Å). The small stacking distance of 3.0 Å in the bb steps might be a consequence of the extended LNA–DNA C–H···O network (Figure 3). However, the actual energetic contribution of the C–H···O contacts is still under debate.[6,7]

To preorganize the sugar moiety for i-motif formation, it must be engineered into a C3′-*endo* conformation. However, most nucleic acid analogues that favor a C3′-*endo* pucker have an electronegative substituent at the 2′-*ribo* position (as in RNA and 2′-*O*-methyl-RNA), which destabilizes or abolishes i-motif formation. With LNA it is possible to combine an oxygen atom at the 2′-*ribo* position with i-motif stability comparable to that of a DNA i-motif. Unfavorable van der Waals contacts in the narrow grooves may be offset energetically by the additional C–H···O contacts, as hinted at in our MD study of **ON2**. The inability of the almost fully modified LNA sequence to form an i-motif may result from the existence of too many unfavorable LNA–LNA contacts in the narrow groove to be offset energetically by the additional C–H···O contacts.

In summary, we have described the ability of LNA-modified oligonucleotides to form i-motifs. These results expand the repertoire of nucleic acid structures in terms of both i-motif formation and LNA oligonucleotides. The modified oligonucleotide **ON2** provides the first example of the stabilization of an i-motif structure by a nucleic acid analogue with a 2′-*ribo* oxygen atom.

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